

**TITLE: TREATMENT OF TISSUE SPECIMENS****Field of the Invention**

This invention relates to the treatment of tissue specimens, particularly (but not exclusively) prior to imaging of the specimens by optical projection tomography (OPT), and to uses of optical projection tomography methods and apparatus.

**Background to the Invention**

Tissue specimens are normally treated by immersion in one or more liquids, before the specimens are imaged in OPT apparatus, and the invention was devised as a way of facilitating this pre-treatment of specimens.

**Summary of the Invention**

According to one aspect of the invention there is provided apparatus for treating tissue specimens by immersion in a liquid, the apparatus comprising a first structure providing a chamber for holding the liquid, and a second structure including holding means for releasably holding the specimens, the first and second structures being relatively moveable in a direction having a vertical component between a first position in which the holding means are relatively close to the chamber and in which the second structure closes the top of the chamber to enable the specimens to be immersed in the liquid whilst the latter is protected from the environment, and a second position in which the holding means are relatively distant from the chamber to enable the specimens to be loaded onto or unloaded from the holding means.

Preferably, the first structure is stationary and the second structure is shiftable vertically with respect to the first structure.

The holding means may include magnets to enable specimens, each provided with a metal mount, to be detachably retained on the second structure by magnetic attraction.

The chamber is preferably in the form of an annular trough in which case the holding means may hold the specimens so that the latter depend from the holding means, conveniently at angularly spaced positions around a circle such that the specimens are lowered into the trough as the second structure is lowered to its first position. In this case, the second structure preferably includes a lid which acts to close the chamber in the first position and the underside of which carries the holding means. Lid closure helps to prevent evaporation of volatile treatment liquids.

The second structure may be rotatably moveable around a central vertical axis, enabling specimens to be loaded onto and unloaded from the second structure at a chosen position alongside the apparatus, either by a robotic arm or a human hand.

The apparatus may have the facility to change the liquid when in the first position, enabling the specimens to be treated by different liquids in a succession of treatment stages, whilst retaining the chamber closed. For example, the apparatus may have a pump to fill and empty the chamber with a succession of chosen liquids which, in the case of tissue specimens, may act to wash or otherwise treat the specimens prior to the specimens being imaged by means of optical projection tomography.

According to another aspect of the invention there is provided a method of treating tissue specimens by immersion in a liquid in a chamber, the method comprising loading the specimens onto a holder so that the specimens depend from the holder and are disposed above the liquid in the chamber, effecting relative movement between the chamber and the specimens in one direction to cause immersion of the specimens in the liquid whilst maintaining the chamber closed and protected from the environment during immersion, effecting relative movement between the chamber and the specimens in the opposite

direction to bring the specimens out of the liquid, and unloading the treated specimens from the holder.

The specimens may be treated by different liquids in a plurality of treatment stages which are preferably carried out by successive emptying and filling of the chamber with the different liquids, whilst the specimens remain in the chamber and whilst the chamber remains closed and protected from the environment.

According to another aspect of the present invention, there is provided a method of performing any one or more of the analyses or procedures listed hereunder comprising use of a method or apparatus of any of the aspects set out above.

There is also provided use of a method or apparatus as described in any of the aspects as set out above in any one or more of the analyses or procedures listed hereunder.

According to the present invention, the analyses and procedures of the present invention include:

Analysis of the structure of biological tissues.

Analysis of the function of biological tissues.

Analysis of the shapes of biological tissues.

Analysis of the distribution of cell types within biological tissues.

Analysis of the distribution of gene activity within biological tissues,

including the distribution of:

- RNA transcripts

- proteins

Analysis of the distribution of transgenic gene activity within biological tissues,

Analysis of the distribution of cell activities within biological tissues,

including:

- Cell cycle status including arrest

- Cell death

- Cell proliferation
- Cell migration

Analysis of the distribution of physiological states within biological tissues.

Analysis of the results of immunohistochemistry staining techniques.

Analysis of the results of in-situ hybridisation staining techniques.

Analysis of the distribution of molecular markers within biological tissues, including any coloured or light-absorbing substances, such as:

5,5'-dibromo-4,4'-dichloro-indigo (or other halogenated indigo compounds)

formazan

or other coloured precipitates generated through the catalytic activity of enzymes

including: b-galactosidase, alkaline phosphatase or other coloured precipitates formed upon catalytic conversion of staining substrates,

including: Fast Red, Vector Red

And including any light-emitting substances,

Therefore including any fluorescent substances,

such as: Alexa dyes, FITC, rhodamine,

And including any luminescent substances,

such as green fluorescent protein (GFP) or similar proteins,

And including any phosphorescent substances.

Analysis of tissues from all plant species.

Analysis of any tissue for agricultural research,

including:

basic research into all aspects of plant biology (genetics, development, physiology, pathology etc.)

analysis of tissues which have been genetically altered.

Analysis of tissues from all animal species.

including:

invertebrates

nematode worms

**vertebrates**

all types of fish (including teleosts, such as zebrafish, and chondryctyes including sharks)

amphibians (including the genus *Xenopus* and axolotls)

reptiles

birds (including chickens and quails)

all mammals (including all rodents, dogs, cats and all primates, including human)

**Analysis of embryonic tissues for any purpose,****including:**

research into any stem cell population

research into developmental biology

research into the causes of abnormal embryo development, including human syndromes

autopsies of human terminated pregnancies (both spontaneous and induced terminations)

**Analysis of any tissues for the purpose of genomics research,****including:**

the analysis of any tissues for the purpose of genomics research,

**including:**

the analysis of transgenic, knock-in, knock-down or knock-out organisms

the analysis or discovery of the expression (or activity) of genes including their spatial distribution, and their levels of expression

the analysis or discovery of abnormalities in the structure or morphology of tissues, as a result of interference due to wilful experimentation (such as genetic or physical modifications including a chemical or biochemical genomics approach), and/or spontaneous abnormalities (such as naturally-occurring mutations)

**Analysis of any tissue for the purpose of neurobiology research,**

including:

- the analysis of the morphology of nerves
- the analysis of the pathways and connectivity of nerves
- the analysis of parts of, or whole, animal brains

**Analysis of any tissue for pharmaceutical research,**

including:

- the analysis of pharmaceutical substances (such as drugs, molecules, proteins, antibodies),
- including their spatial distribution within the tissue, and their concentrations
- the analysis or discovery of abnormalities in the structure or morphology of tissues.

**Analysis of tissues for medical research,**

including:

- research into the genetics, development, physiology, structure and function of animal tissues
- analysis of diseased tissue to further our understanding of all types of diseases

including:

congenital diseases

acquired diseases

including:

infectious

neoplastic

vascular

inflammatory

traumatic

metabolic

endocrine

degenerative

drug-related

iatrogenic or

**idiopathic diseases****Analysis of tissues for medical diagnosis, treatment or monitoring,****including:****the diagnosis of cancer patients****including:****searching for cancerous cells and tissues within biopsies****searching for abnormal structure or morphology of tissues within biopsies****the analysis of all biopsies****including the analysis of:****lymph nodes****polyps****liver biopsies****kidney biopsies****prostate biopsies****muscle biopsies****brain tissue****the analysis of tissue removed in the process of extracting a tumour from a patient****including:****determining whether all the tumour has been removed****determining the type of tumour, and the type of cancer.**

According to the present invention, samples for use in the present invention may be prepared as described in the earlier patent applications and/or employing conventional pathological and histological techniques and procedures well known to persons skilled in the art.

For example, in-situ hybridisation (particularly useful for detecting RNAs):Hammond K L, Hanson I M, Brown A G, Lettice L A, Hill R E "Mammalian and Drosophila dachsund genes are related to the Ski proto-oncogene and are expressed in eye and limb". Mech Dev. 1998 Jun;74(1-2):121-31.

Immunohistochemistry (particularly useful for detecting proteins and other molecules): Sharpe J, Ahlgren U, Perry P, Hill B, Ross A, Hecksher-Sorensen J, Baldock R, Davidson D. "Optical projection tomography as a tool for 3D microscopy and gene expression studies" *Science*. 2002 Apr 19;296(5567):541-5.

It will be appreciated that modification may be made to the invention without departing from the scope of the invention.

#### **Brief Description of the Drawings**

The invention will now be further described, by way of example, with reference to the accompanying drawings, in which:-

**Figure 1** is an isometric view of apparatus according to the invention and in an open condition,

**Figure 2** is an isometric view of the apparatus in a closed condition,

**Figure 3** is a fragmentary view, to an enlarged scale, of part of the apparatus in an open condition, showing specimens held by the apparatus,

**Figure 4** is a cross-sectional view of the apparatus,

**Figure 5** shows the apparatus in combination with a robotic arm,

**Figure 6** is an enlarged view of part of Figure 5, and

**Figure 7** shows the robotic arm positioning a specimen onto the rotary stage of an OPT scanner.

### Detailed Description

The apparatus comprises a fixed structure 1 having a cylindrical outer casing 2 in the top of which is positioned an annular trough 3 which is open at the top. Mounted in the fixed structure is a moveable structure 4 having a central hub or spindle 5 on the top of which is mounted a disc-like lid 6 the underside of which carries a number of angularly spaced and downwardly depending cylindrical magnets 7. The hub or spindle 5 is rotatably mounted in the fixed structure for rotation of the moveable structure about a central vertical axis indicated at 8 in Figure 4. Also, the hub or spindle 5 is capable of vertical translational movement with respect to the fixed structure 1 along the vertical axis 8.

Within the casing 2 are located motors and gearing for driving the structure 4 both in rotation and translational movement, as indicated at 9 in Figure 4. The trough 3 is capable of being filled with liquids, and the structure 1 includes containers for holding these liquids and pumps for filling and emptying the trough, as indicated at 10 in Figure 4.

The magnets 7 are used to hold, in a detachable manner, tissue specimens 12 each of which has been prepared with a metal mount 13 at one end of the specimen. This allows each metal 13 mount to depend from one of the magnets 7, with the specimen 12 depending downwardly from the mount 13. When the apparatus is in the open condition (Figure 1), the magnets 7 are raised clear of the top of the trough 3 so that the specimens 12 can be attached to or removed from the magnets 7. When the apparatus is in the closed position (Figure 2), the lid 6 engages the top of the casing 2 and the specimens 12 are immersed in a liquid 14 in the trough 3.

The robotic arm 15 shown in Figures 6 and 7 is used to load untreated specimens into the apparatus and also to transfer treated specimens from the apparatus to the rotary stage 16 of an OPT scanner where the specimens are imaged.

In use, the robotic arm 15 is used to load specimens into the apparatus, each specimen being attached to the lower end of a corresponding magnet 7 by virtue of the magnetic attraction between the magnet 7 and the metal mount 13 at one end of the specimen 12, the moveable structure 4 being in the open position and being indexed in a rotational sense as the specimens are loaded. When loaded with specimens, the moveable structure is moved to its lowered or closed position, thereby immersing the specimens 12 in the liquid 14 which has been pumped into the trough 3. In this closed position, the lid 6 engages the upper rim of the outer casing 2 so that the liquid 14 is closed to the air, thereby allowing the use of a volatile liquid, unlike known apparatus which uses conveyor belts for transferring specimens through a liquid.

If it is required to treat the specimens by a succession of liquids, the first liquid is drained from the trough 3 and a second liquid pumped thereinto, without the need for the trough 3 to be opened to the air. Moreover, the attachment of the metal mounts 13 to the magnets 7 retains the specimens 12 in their hanging positions so that the specimens do not engage the bottom of the trough 3, which could damage them.

Any number of treatment stages can be carried out in this manner, the liquid being changed without the need to move the specimens and employing only a small volume of each treatment liquid.

After treatment of the specimens 12, the moveable structure 4 is raised to its upper position and the treated specimens 12 are then transferred to the rotary stage 16 by means of the robotic arm 15, the structure 4 being rotationally indexed to enable the robotic arm 15 to unload each specimen 12 in turn and to transfer the treated specimen 5 to the rotary stage 16.

Examples of liquids for treating the specimens are fixatives (such as paraformaldehyde or formalin), alcohols (in particular methanol and ethanol) and organic solvents for clearing the specimens (in particular benzyl alcohol and benzyl benzoate).

The apparatus and methods can be used in various analyses and procedures, as set out below:

**Analysis of the structure of biological tissues.**

**Analysis of the function of biological tissues.**

**Analysis of the shapes of biological tissues.**

**Analysis of the distribution of cell types within biological tissues.**

**Analysis of the distribution of gene activity within biological tissues,**  
**including the distribution of:**

- RNA transcripts
- proteins

**Analysis of the distribution of transgenic gene activity within biological tissues,**

**Analysis of the distribution of cell activities within biological tissues,**  
**including:**

- Cell cycle status including arrest
- Cell death
- Cell proliferation
- Cell migration

**Analysis of the distribution of physiological states within biological tissues.**

**Analysis of the results of immunohistochemistry staining techniques.**

**Analysis of the results of in-situ hybridisation staining techniques.**

**Analysis of the distribution of molecular markers within biological tissues,**  
**including any coloured or light-absorbing substances,**

**such as:**

5,5'-dibromo-4,4'-dichloro-indigo (or other halogenated indigo compounds)  
formazan

or other coloured precipitates generated through the catalytic activity of enzymes  
including:  $\beta$ -galactosidase, alkaline phosphatase or other coloured precipitates  
formed upon catalytic conversion of staining substrates,  
including: Fast Red, Vector Red

And including any light-emitting substances,  
Therefore including any fluorescent substances,  
such as: Alexa dyes, FITC, rhodamine,  
And including any luminescent substances,  
such as green fluorescent protein (GFP) or similar proteins,  
And including any phosphorescent substances.

**Analysis of tissues from all plant species.**

**Analysis of any tissue for agricultural research,**

including:

basic research into all aspects of plant biology (genetics, development, physiology, pathology etc.)  
analysis of tissues which have been genetically altered.

**Analysis of tissues from all animal species,**

including:

invertebrates

nematode worms

vertebrates

all types of fish

(including teleosts, such as zebrafish, and chondrycthes including sharks)

amphibians (including the genus *Xenopus* and axolotls)

reptiles

birds (including chickens and quails)

all mammals (including all rodents, dogs, cats and all primates, including human)

**Analysis of embryonic tissues for any purpose,**

including:

research into any stem cell population

research into developmental biology

research into the causes of abnormal embryo development, including human syndromes

autopsies of human terminated pregnancies (both spontaneous and induced terminations)

Analysis of any tissues for the purpose of genomics research,

including:

the analysis of transgenic, knock-in, knock-down or knock-out organisms

the analysis or discovery of the expression (or activity) of genes including their spatial distribution, and their levels of expression

the analysis or discovery of abnormalities in the structure or morphology of tissues, as a result of interference due to wilful experimentation (such as genetic or physical modifications including a chemical or biochemical genomics approach), and/or spontaneous abnormalities (such as naturally-occurring mutations)

Analysis of any tissue for the purpose of neurobiology research,

including:

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the analysis of the pathways and connectivity of nerves

the analysis of parts of, or whole, animal brains

Analysis of any tissue for pharmaceutical research,

including:

the analysis of pharmaceutical substances (such as drugs, molecules, proteins, antibodies),

including their spatial distribution within the tissue, and their concentrations

the analysis or discovery of abnormalities in the structure or morphology of tissues.

Analysis of tissues for medical research,

including:

research into the genetics, development, physiology, structure and function of animal tissues

analysis of diseased tissue to further our understanding of all types of diseases including:

congenital diseases

acquired diseases

including:

infectious

neoplastic

vascular

inflammatory

traumatic

metabolic

endocrine

degenerative

drug-related

iatrogenic or

idiopathic diseases

Analysis of tissues for medical diagnosis, treatment or monitoring,

including:

the diagnosis of cancer patients

including:

searching for cancerous cells and tissues within biopsies

searching for abnormal structure or morphology of tissues within biopsies

the analysis of all biopsies

including the analysis of:

lymph nodes

polyps

liver biopsies

kidney biopsies

**prostate biopsies**

**muscle biopsies**

**brain tissue**

**the analysis of tissue removed in the process of extracting a tumour from a patient including:**

**determining whether all the tumour has been removed**

**determining the type of tumour, and the type of cancer.**

**It will be appreciated that modification may be made to the invention without departing from the scope of the invention.**